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## **Intravesical oncolytic viral therapy using attenuated, replication-competent, herpes simplex viruses G207 and NV1020 is effective in the treatment of bladder cancer in an orthotopic syngeneic model**

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### **ABSTRACT**

Attenuated, replication-competent herpes simplex virus mutants are attracting interest because of their ability to replicate within and kill tumor cells while remaining of low pathogenicity to normal tissues. In this study we investigated the ability of two oncolytic candidates, G207 and NV1020, to infect and lyse human and murine transitional cell carcinoma (MBT-2) cells *in vitro* and their *in vivo* efficacy in a well-established immunocompetent animal model of bladder cancer. Both viruses were effective at infecting, replicating within, and achieving subsequent cell lysis for all four human bladder cancer cell lines and MBT-2. We found a strong correlation between infection efficiency and subsequent cell death. *In vivo* studies demonstrated that these viruses were effective with a single intravesical instillation and even more effective with multiple instillations at reducing tumor burden and achieving cures of orthotopic bladder cancer in syngeneic C3H/HeJ mice. Immunohistochemistry and histological studies demonstrated that viral replication and cell death were restricted to bladder cancer cells. These results suggest that both G207 and NV1020 hold particular promise for intravesical treatment of human bladder cancer and that ease of intravesical instillation facilitates efficient delivery of virus to tumor cells.

**Key Words:** G207 • NV1020 • orthotopic bladder cancer • oncolytic viral therapy

**I**t is estimated that in 1999, 54,000 cases of bladder cancer occurred in the USA, resulting in more than 12,000 deaths (1). Patients with tumor superficial to the muscle layer of the bladder are usually treated by endoscopic resection alone. Unfortunately, these patients have

a 50–70% recurrence rate within 1–2 years and a progression rate of up to 38% (2). Attempts to reduce the risk of recurrence and progression of superficial bladder cancer have included the administration of intravesical immunotherapy with intravesical bacillus Calmette-Gueurin (BCG) or chemotherapy with limited results (3–8). Approximately 30% of patients will present with tumors invading the muscle layers of the bladder. These patients are at high risk for tumor dissemination; and despite aggressive modern therapy, including early radical cystectomy, the overall 5-year survival rate continues to be less than 50% (2). Large randomized controlled trials of neoadjuvant chemotherapy prior to cystectomy, using currently available drug regimens, have failed to benefit patients, and no conclusive evidence exists for the efficacy of adjuvant chemotherapy (2, 9). Clearly, there is a need to develop new treatment strategies for bladder cancer, particularly for high-risk superficial tumors, BCG failures, and muscle-invasive cancers. There has been much recent interest in the treatment of various experimental cancers with replication-competent viruses (10–12). Mutant viruses based on herpes simplex virus 1 (HSV-1) have been engineered that are attenuated for virulence against *normal* tissues but retain the ability to replicate in cancer cells causing a lytic infection and subsequent cell death (13–16).

G207 is a genetically engineered oncolytic virus based on wild-type herpes simplex type-1 (17, 18). The key features of G207 include the deletion of both copies of  $\gamma_134.5$  leading to highly attenuated neurovirulence and interruption of the  $U_L39$  gene, which encodes for the large subunit of ribonucleotide reductase, an enzyme required for viral DNA synthesis in nondividing cells (19). The  $U_L39$  gene interruption was achieved by insertion of a *lacZ* gene, which allows  $\beta$ -galactosidase expression to be determined as a measure of viral infectivity.

NV1020 is a nonselected clonal derivative of R7020, originally constructed for vaccine studies against HSV-1 and HSV-2 (20). NV1020 has a 700-bp deletion in the thymidine kinase locus and a 15-kb deletion across the joint region of the long (L) and short (S) components of the HSV-1 genome. The L/S junction of the NV1020 contains a 5.2-kb fragment of HSV-2 DNA inserted for previous vaccine studies and an exogenous copy of the thymidine kinase gene under the control of the HSV-1  $\alpha 4$  promoter. NV1020/R7020, which has an excellent safety profile in rodents (20) and nonhuman primates (21), has demonstrated oncolytic activity in human head and neck cancer cells (22).

This study investigates the safety and efficacy of G207 and NV1020 *in vitro* and *in vivo* in an orthotopic syngeneic model of bladder cancer and compares the efficacy of these oncolytic viruses.

## METHODS

### Cell lines, viruses, and BCG

Murine bladder tumor-2 (MBT-2), a FANFT [N[4-(5-nitro-2 furyl)-2 thiozoly] formamide] induced bladder cancer cell line originally isolated and characterized in syngeneic C3H/HeJ mice (23, 24), was kindly donated by Timothy Ratliff (University of Iowa, Iowa City). The human transitional cell carcinomas, T-24 (25, 26) and J-82 (27), and the human transitional cell papilloma RT-4 (25, 26) were obtained from the American Type Culture Collection (ATCC) (Rockville, Md.). SKUB cells were recently isolated and characterized at Memorial Sloan-Kettering Cancer Center from a primary transitional cell carcinoma of the bladder. RT-4 cells

were cultured in McCoy's 5a medium supplemented with 10% heat-inactivated fetal calf serum (IFCS) (HyClone, Logan, Utah) and 1% L-glutamine. J-82 cells were cultured in Eagle's minimum essential medium in Earle's BSS with nonessential amino acids supplemented with 10% IFCS and 1% L-glutamine. MBT-2, T-24, and SKUB cell lines were maintained in RPMI 1640 supplemented with 10% IFCS and 1% L-glutamine. Vero cells (African green monkey kidney cells) were obtained from the ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM) with 5% IFCS. All cell lines were maintained at 37°C in 5% CO<sub>2</sub> humidified atmosphere. For implantation into animals, subconfluent cells were washed in PBS, trypsinized with 0.25% trypsin, and centrifuged at 7000 rpm for 5 min in a tabletop Sorvall (Kendro Laboratory Products, Newtown, Conn.). Cells were then resuspended in PBS and immediately implanted orthotopically into the animals.

G207, a kind gift from S. Rabkin and B. Martuza, was constructed as previously described (17). It deleted for both copies of the  $\gamma_{134.5}$  gene and contains an *Escherichia coli lacZ* gene insertion inactivating the U<sub>L</sub>39 gene of the R3616 mutant. Virus was propagated on Vero cells at a multiplicity of infection (MOI) of 0.02 at 34°C, harvested after 2 days and subjected to freeze thaw lysis and sonication to release G207 from the cell fraction as previously described (28). Cell debris was removed by centrifugation (300 g for 10 min at 4°C). Viral titers were determined by standard plaque assay on Vero cells, and virus was stored at -80°C in NaCl-20mM Tris (pH 7.5).

NV1020 derived from a nonselected clone of R7020, a genetically engineered attenuated strain of HSV-1 (20). Stocks of NV1020 were produced in WHO Vero cells seeded in roller bottles and infected at an MOI of 0.01. Virus was purified by size exclusion chromatography (°500, Amersham Pharmacia Biotech), concentrated by ultrafiltration (0.05 µM pore size, polysulfone hollow-fiber membrane, Spectrum), and formulated in D-PBS/10% glycerin.

Connaught strain BCG (Theracys, Connaught Laboratories, Toronto, Ontario, Canada) was obtained as a freeze-dried suspension and reconstituted in the diluent provided according to the manufacturer's instructions to achieve a final concentration of  $1.2 \times 10^7$  colony-forming units (CFU)/50 µl for treatment of each animal.

### ***In vitro* assay of infection and lysis of human and murine transitional cell carcinoma**

Infectivity experiments were conducted with G207, which expresses the *lacZ* gene (using X-Gal/X-Gal histochemistry), to measure the infection efficiency of the cancer cells. All five cell lines were plated in triplicate in 24 well plates (Becton Dickinson, Lincoln Park, N.J.) at  $5 \times 10^4$  cells/well. Within one doubling time (i.e., within 24 h), media was removed from all wells and fresh media was added to control wells, and media-infected with heat inactivated (45 min at 55°C) or active G207 at MOIs of 0.01, 0.1, 1, and 2 was added to the remaining wells. At 24, 48, 72, 96, and 120 h, media was removed, wells were washed with PBS, and cells were exposed to 0.25% trypsin. Cell counts were performed, in triplicate, on a hemocytometer using trypan blue exclusion. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining was performed in triplicate on identically prepared serial plates at 24, 48, and 72 h, and percent blue cells were calculated as a measure of infectivity of G207. Percent blue cells were calculated by averaging blue cells present in three high-power fields, for each well, in triplicate, for each cell line. This method allowed nine observations to be made for each cell line, at each MOI and at each time

point examined. After we established the *in vitro* efficacy and infectivity of G207, we performed experiments to directly compare G207 and NV1020 for all five cell lines. In this group of experiments,  $4 \times 10^4$  cells were plated in triplicate into 24 well plates in 1 ml of media. Within 24 h, media was removed from all wells and fresh media was added to control wells, and media infected with G207 and NV1020 at MOIs of 0.01, 0.1, and 1 was added to the remaining wells. Cell counts were performed as described above at 24, 48, 72, and 96 h.

### **Viral growth curves**

To determine viral replication in the various cell lines, supernatants were collected prior to X-Gal staining of plates from the G207 experiment, and a viral titer assay was performed by counting plaque-forming units (PFUs) on Vero cells. All five cell lines were examined, in triplicate, for their ability to support replication of G207 at MOIs of 0.01, 0.1, 1, and 2 at 24, 48, and 72 h. Supernatants from heat-inactivated and control wells were assayed on Vero cells in parallel.

Cell lines were also examined and compared for total viral production *in vitro* after exposure to G207 or NV1020. In addition,  $5 \times 10^5$  cells were plated in duplicate for each cell line (RT-4 and J-82) in 6-well plates (Costar Corporation, Cambridge, Mass.) and infected with G207 or NV1020 at an MOI of 0.01, with uninfected wells serving as negative controls. At each time point (daily up to day 8 for J-82 and up to day 5 for RT-4), cells were scraped from the well using Cell Lifters (Costar Corporation, Cambridge, Mass.). Total well contents were collected into 15-ml polypropylene conical tubes (Becton Dickinson Labware, Franklin Lakes, N.J.) and subjected to four cycles of freeze-thaw lysis, sonication, and centrifugation at 3000 rpm for 10 min at 4°C. Supernatants were diluted appropriately and plated on confluent Vero cells in 6-well plates. After 2 h, Vero cells were covered with 2% agarose in DMEM, 10% FCS to restrict all viral spread except for direct cell-to-cell contact. Viral plaques were counted 3 days after infection, and viral titers were determined.

### **Determination of cell-doubling time and S-phase fraction**

Cells measuring  $5 \times 10^4$  were plated in triplicate into T-25 flasks (Fischer Scientific Co., Agawam, Mass.) in 10 ml of medium. The cell monolayer was trypsinized with 0.25% trypsin and viable cells counted by trypan blue exclusion using a hemocytometer at 24-h intervals. Data was plotted for log-phase growth, and doubling time was determined. Cells were prepared for cell cycle analysis using the nuclear preparation techniques described by Nusse et al. (29, 30). Briefly, subconfluent flasks of MBT-2 cells were trypsinized, washed in PBS, and digested in DNase-free RNase (10 mg/ml, Boehringer Mannheim, Indianapolis, Ind.), for 60 min at room temperature and then stained with ethidium bromide (10 mg/ml, Gibco BRL, Life Technologies Inc., Gaithersburg, Md.). Cell cycle analysis was performed on a FACScan equipped with a FACStation running CellQuest software (Becton Dickinson, San Jose, Calif.). A forward-angle, light-scatter threshold trigger was used to eliminate debris. Cell clumps were removed using analysis gates on either fluorescence pulse width or height versus pulse area (integral).

### **Animal studies**

Approval from the Institutional Animal Care and Use Committee was obtained before conducting animal experiments. Female C3H/HeJ mice were obtained from Jackson Laboratories (Bar Harbor, Maine). They were housed in standard laboratory conditions and fed laboratory chow and water *ad libitum*. Previous investigators have recommended orthotopic implantation of MBT-2 cells into C3H/HeJ as an excellent model for human bladder cancer (23, 24, 31, 32, 36, 37). Previous experiments have demonstrated that more than 95% of 6-month-old animals will develop bladder tumors after intravesical inoculation of  $5 \times 10^5$  MBT-2 cells without the need for mucosal injury by electrocautery and that these tumors will be superficial by day 10 and muscle invasive by day 20. For all *in vivo* experiments, animals were anesthetized with a single intraperitoneal dose consisting of Ketamine (90 mg/kg, Fort Dodge Animal Health, Fort Dodge, Iowa) and Xylazine (9 mg/kg, Bayer Corp., Animal Health, Shawnee Mission, Kans.). Animals were then catheterized, per urethra, with a 24G catheter (Angiocath™, Becton-Dickinson Infusion Therapy Systems, Sandy, Utah). Instillation of MBT-2 ( $5 \times 10^5$ ) cells and treatment with virus was achieved by suspending cells or virus in 50  $\mu$ l of PBS in a 1-ml syringe and ensuring that the solution remained in the bladder for at least 1 h. Animals were inspected daily for signs of excessive tumor burden, including a palpable lower abdominal mass and hematuria, and weighed on alternate days. Animals were randomized into treatment groups.

### **In vivo assay of infectivity and dissemination**

#### ***Histochemical analysis***

G207 was chosen for these experiments because the *lacZ* gene allows identification of infected cells. A total of 22 animals were used for *lacZ* expression studies. Initially, animals at day 8 ( $n=4$ ), day 12 ( $n=4$ ), and day 16 ( $n=5$ ) following tumor instillation and animals without tumor ( $n=4$ ) were treated with a single intravesical instillation of  $1 \times 10^7$  PFUs of G-207. To examine the influence of dose of virus on *lacZ* expression, on day 12 following orthotopic tumor implantation, additional animals were treated with  $6 \times 10^5$  ( $n=2$ ),  $6 \times 10^6$  ( $n=2$ ), and  $1 \times 10^8$  ( $n=1$ ) PFU. These animals were euthanized 48 h following treatment; and organs including bladder, liver, brain, kidney, spleen, lung, ovary, and heart were harvested and frozen in Tissue-Tek OCT Compound (Sakura Finetek, Torrance, Calif.) and stored at  $-80^\circ\text{C}$ . They were subsequently sectioned by cryotome. Sections of 8  $\mu$ m were fixed in 1% glutaraldehyde and stained with X-Gal solution to evaluate  $\beta$ -galactosidase expression. Sections were counterstained with Nuclear Fast Red (Sigma, St. Louis, Mo.). Semiquantitative evaluation of  $\beta$ -galactosidase expression in tumor and normal urothelium was undertaken, and the results were expressed in quartiles as a percentage of tumor cells that stained blue.

#### ***Quantitative PCR analysis***

To further define the persistence of infection and the extent of dissemination, we performed quantitative PCR analysis on genomic DNA extracted from bladder, liver, lung, brain, kidney, testes, and serum of animals treated at day 12 after tumor instillation with G207 or NV1020. At day 1 ( $n=4$ ), day 7 ( $n=4$ ), and day 14 ( $n=2$ ) postbladder irrigation with  $1 \times 10^7$  PFU of G207 or NV1020, tissues were obtained. Genomic DNA extraction was performed on all tissues. Standard curves were established by doping uninfected liver and serum with known quantities of the G207 virus prior to DNA extraction. Real-time quantitative PCR was performed using an ABI Prism 7700 Sequence Detector (Perkin Elmer Biosystems, Foster City, Calif.), as previously

described (28). Sense (5'-ATGTTTCCCGTCTGGTCCAC-3') and antisense (5'-CCCTGTCGCCTTACGTGAA-3') primers, and a dual labeled fluorescent TaqMan probe (5'-6FAM-CCCGCTCTCCATGTCCAGGATGG-TAMRA-3') were designed to span the 111-bp fragment of the HSV ICP0 (immediate early gene). Additional sense (5'-CGCCTACCACATCCCAAGGAA-3') and antisense (5'-GCTGAATTACCGCGGCT-3') primers and TaqMan probe (5'-JOE-TGCTGGCACCAGCTTGCCCTC-TAMRA-3') for the 187-bp 18S rRNA coding sequence were used in the same reaction to normalize the amount of total DNA. Samples were subjected to 40 cycles of PCR (stage 1, 50 °C -2 min; stage 2, 95 °C—10 min; stage 3, 95 °C—15 s and 60 °C—1 min; and stage 4, 25 °C—soak). The AmpliTaq Gold nuclease cleaves a fluorescent dye (FAM or JOE) from the nonextendable probe, liberating it from the proximity of an associated quencher (TAMRA). Probe binding is a requisite for primer extension. The sequence detector is coupled to a charge-coupled device camera, which records the fluorescent emission spectra from individual wells at 500–650 nm with each cycle. Specificity for amplified product is conferred by both probe and primer sets, obviating the need for Southern blot analysis of the PCR product.

### ***Histologic analysis***

Animals at day 3 following tumor inoculation were treated with  $1 \times 10^7$  PFU of intravesical G207 or NV1020 and were euthanized in groups of 3, for each virus, at day 1 and day 7. Brain, liver, and kidney were harvested and fixed in formalin. Standard hematoxylin and eosin staining was performed to evaluate any signs of inflammation in these organs.

### ***In vivo efficacy of intravesical oncolytic viral therapy***

To compare control and G207, animals were treated with a single instillation of 50  $\mu$ l PBS (C) or G207 ( $1 \times 10^7$  PFU in 50  $\mu$ l PBS) at day 3 following tumor inoculation. Three experiments with five animals in each study group were used. Results were very similar and were combined for analysis ( $n=30$  total;  $n=15$  in each group). Our experiments and those of other investigators have confirmed the equivalence of heat-inactivated G207 and heat-inactivated NV1020 with control (17, 22, 28, 33–35). Animals were terminated at day 19 because of signs of excessive tumor burden in the control group.

To compare control, G207, NV1020, and BCG, animals were treated weekly for 3 weeks beginning at day 3 following tumor inoculation. Two experiments were performed; results were very similar and were combined for analysis. A total of 50 animals were used. Animals were treated with 50  $\mu$ l PBS (control,  $n=12$ ),  $1 \times 10^7$  PFU G207 ( $n=13$ ),  $1 \times 10^7$  PFU NV1020 ( $n=13$ ) or  $1.2 \times 10^7$  CFU BCG ( $n=12$ ) in 50  $\mu$ l PBS. The dosage regimen was chosen because previous investigators have shown it to be the most efficacious regimen for cytokine induction and antitumor activity following intravesical BCG in this animal model (36, 37). Animals in this experiment therefore received three treatments at weekly intervals before termination. Animals were terminated at day 21 following tumor inoculation because the control animals exhibited signs of excessive tumor burden.

End-points in these experiments included differences among groups in weight loss, tumor weight, and the number of animals without bladder tumor at necropsy. Bladders were opened and inspected, tumors were weighed, and bladders were fixed, sectioned, and examined

histologically for any tumors not visible macroscopically. Experiments ended when control animals exhibited signs of excessive tumor burden (e.g., hematuria, palpable lower abdominal mass, and weight loss in excess of 10% of starting body weight). Full necropsies were performed, and bladder, kidney, liver and lung were harvested for histological examination. Animals were defined as tumor free if no macroscopic or microscopic (histological) evidence of tumor was identified. Animals were randomized into treatment groups, and control animals were treated in identical fashion to treated animals but received 50  $\mu$ l of intravesical PBS. Initial experiments were conducted with G207 to allow X-Gal staining of the bladder tumors.

### Statistical analysis

Wilcoxon Signed Ranks test was used to analyze for differences between results of the cell viability assay. Least-squares linear regression was used to analyze for a correlation between cell survival and  $\beta$ -galactosidase activity. The unpaired *t*-test (2-tailed) and the Fischers Exact Test were used to analyze differences between treated groups *in vivo*. SPSS version 9.0.0 for Windows (www.spss.com) and Graph Pad Instat version 3.01 for Windows (Graphpad Software, San Diego, Calif., www.graphpad.com) software were used.

## RESULTS

### *In vitro* assay of infection and lysis of human and murine transitional cell carcinoma

G207 proved highly effective at an MOI = 2 with 100% *in vitro* cell lysis at 5 days following infection for all 5 cell lines (data not shown). Both G207 and NV1020 demonstrated highly effective cell lysis for all cell lines at MOI =1, but no statistically significant difference between the efficacy of the viruses was demonstrated (Fig. 1). A statistically significant difference was demonstrated at MOI 0.1, and NV1020 proved more efficacious than G207 in the poorly differentiated human transitional cell carcinoma cell lines J-82 ( $P<0.004$ ), SKUB ( $P=0.02$ ), and T-24 ( $P<0.001$ ) (Fig. 2). At an MOI of 0.01, both viruses were ineffective for MBT-2, J-82, and T-24; however, an effect was observed for RT-4 and SKUB, but no significant difference between viruses was demonstrated (data not shown). No difference between the viability of control cultures or cells infected with the heat-inactivated viruses was demonstrated throughout these experiments (data not shown).

In addition, G207 demonstrated very impressive infection efficiency, as measured by the percentage of cells expressing  $\beta$ -galactosidase (percent blue cells) in all cell lines tested. Infection efficiency of 100% was seen with G207 at an MOI of 1 at 24 h in MBT-2, SKUB, T-24, and RT-4 cells and at an MOI of 2 at 48 h in J-82 cells. The percentage of blue cells increased from 24 h through to 72 h for all cell lines, and a greater percentage of blue cells was seen at the latter time points (data not shown). The RT-4 cell line was the cell line that was most susceptible to infection by G207. Even at the lowest MOI of 0.01, impressive X-Gal staining was demonstrated at the latter time points (Fig. 3). A correlation was found between infection efficiency and cytotoxicity for all cell lines ( $R=0.9$ , least-squares linear regression).

### Viral growth curves

It is clear that viral replication is supported by bladder cancer cells. RT-4 and J-82 were the most and least supportive, respectively, of replication of G207 and NV1020 (Fig. 4). This is not surprising, because these are also the most susceptible (RT-4) and least susceptible (J-82) cell lines for G207 and NV1020 cytotoxicity. When compared directly, NV1020 replicated to higher titers than G207. For the most permissive cell line, RT-4, peak viral titers were  $20.8$  and  $40.0 \times 10^6$  PFU, respectively, for G207 and NV1020, which is a 4160- and 8000-fold increase in viral titer. Even for the least permissive cell line, J-82, there was evidence of viral replication. Peak viral titer in the J-82 cell line was significantly greater for NV1020 at day 6 following treatment than for G207 ( $23.4$  vs.  $0.05 \times 10^6$  PFU,  $P < 0.001$ ). This is still 4680 and 10-fold increase from original titers.

### **Determination of cell-doubling time and S-phase fraction**

No correlation was found between the oncolytic effect of the viruses and S-phase fraction or doubling time. However, all cell lines had rapid doubling times between 22 and 30.5 h (Table 1). Furthermore, all 5 cell lines examined in this study were very susceptible to the oncolytic effect of these viruses (Fig. 1).

### ***In vivo* assay of infectivity and dissemination**

#### **Histochemical analysis**

In the animals with day 12 tumors,  $\beta$ -galactosidase expression was clearly identified in tumor but not normal urothelium 48 h after treatment with G207. Blue cells were readily observed at each dose of  $6 \times 10^5$  ( $n=2$ ),  $6 \times 10^6$  ( $n=2$ ), and  $1 \times 10^8$  ( $n=1$ ) PFU. An intermediate dose of  $1 \times 10^7$  PFU was chosen to continue experiments and was administered to animals at day 8 ( $n=4$ ), day 12 ( $n=4$ ), and day 16 ( $n=5$ ) following tumor instillation and to animals without tumor ( $n=4$ ). Blue cells were identified within 60–75% of bladder tumors at each time point. In tumors with staining, the percentage of tumor cells stained varied between 25% and 50%, and no staining of normal urothelium was observed (Fig. 5). In animals without tumor, very sparse staining of normal urothelium ( $<1\%$ ) was identified. In multiple experiments, no blue cells were observed in liver, brain, kidney, spleen, lung, ovary, and heart harvested from these animals.

#### **Quantitative PCR analysis**

To further define the presence, persistence, and extent of dissemination of G207 and NV1020 after bladder irrigation, quantitative PCR analysis of tissue and serum samples was performed. By establishing a standard curve with 1, 5, 10,  $10^2$ , and  $10^3$  PFU, we first determined our limit of reliable detection as 10 PFU per 50 mg of tissue. When the various organs (bladder, liver, lung, kidney, and brain) were analyzed, only bladder clearly demonstrated the presence of the HSV-ICP0 DNA sequence at any time point evaluated. Furthermore, bladder exposed to irrigation with G207 or NV1020 demonstrated peak appearance of viral DNA on day 1 (2000 and 2500 viral genomic equivalents per 1  $\mu$ g genomic DNA for G207 and NV1020, respectively), decreasing at day 7 (1200 and 1500), but still detectable at 14 days (500 and 500). Further analysis of tumor specimens dissected from bladder reveals that most of the recovered viral DNA is associated with the tumor specimens.



## Histologic analysis

Standard hematoxylin and eosin staining performed on brain, liver, and kidney harvested from animals treated at day 1 and day 7 with  $1 \times 10^7$  PFU of intravesical G207 or NV1020 revealed normal histology for these organs and, in particular, no evidence of inflammation was observed.

## *In vivo* efficacy of intravesical oncolytic viral gene therapy

Animals treated with a single instillation of 50  $\mu$ L PBS (C) or G207 ( $1 \times 10^7$  PFU in 50  $\mu$ L PBS) at day 3 following tumor inoculation ( $n=15$  in each group) showed a significant reduction in tumor weight in the G207 treated group ( $P=0.02$ , Mann-Whitney U test). All 15 animals in the control group developed tumors with a mean weight ( $\pm$ SD) of 310 ( $\pm$ 130) mg compared with 9 animals in the G207 group with a mean tumor weight ( $\pm$ SD) of 150 ( $\pm$ 110) mg. No bladder tumor was found in four animals in the G207 group examined macroscopically and microscopically at necropsy. Control animals exhibited signs of excessive tumor burden, including weight loss, hematuria, and a palpable mass, whereas treated animals continued to groom themselves and maintain weight. Animals tolerated the treatment well with no adverse events and no deaths.

Both G207 and NV1020 were highly effective when administered weekly for 3 weeks (Table 2). Treatment was well tolerated with no adverse events and no treatment-related deaths. Treated animals continued to demonstrate normal activity with normal feeding and grooming habits. Cured animals maintained weight, but treated animals that were not cured experienced weight loss similar to that of control animals. Animals were terminated at day 21 because of signs of excessive tumor burden in the control group.

Ten of 11 animals in the control group demonstrated bladder tumors at necropsy. Six of 12 animals in the BCG group demonstrated bladder tumors ( $P=0.06$ ). A significant difference was seen in the groups treated with virus when compared with control with 5 of 13 animals in the G207 group ( $P=0.02$ ) and only 2 of 12 animals in the NV1020 group with bladder tumors ( $P=0.001$ ). Tumor weight was also significantly reduced between groups treated with virus when compared with control ( $P=0.001$  for NV1020 and  $P=0.008$  for G207). There was no statistically significant difference between viruses and similarly, there was no difference when comparing NV1020 and BCG or G207 and BCG (Table 2).

## DISCUSSION

More effective treatments are required for patients with high-risk superficial bladder cancer. The results of intravesical chemotherapy in this group of patients were disappointing: The recurrence rate dropped by only 14%, and there was no effect on disease progression (6). Intravesical bacillus Calmette-Geurin (BCG), the most effective treatment to date, has helped to reduce disease recurrence (3, 6, 8). However, excessive morbidity and even mortality from intravesical BCG, together with recent reports demonstrating no effect on the long-term risk of disease progression, suggest that intravesical BCG may not be the optimal treatment for patients with high-risk superficial bladder cancer (4, 5). Currently, patients with high-risk superficial disease (high-grade T1 or carcinoma *in situ*) who fail intravesical BCG must undergo radical surgery

because there are no effective alternative treatments. These experiments evaluate the efficacy of multimutated, attenuated, replication-competent oncolytic viruses G207 and NV1020 for the treatment of transitional cell carcinoma *in vitro* and *in vivo* in the best animal model of human bladder cancer currently available.

The current studies demonstrate that these viruses are promising agents in the treatment of bladder cancer. G207 and NV1020 are able to infect, replicate, and lyse preferentially in rapidly dividing cells. Both G207 and NV1020 have recently been found to be effective in treating various experimental cancers while maintaining an excellent safety profile (22, 28, 33–35). Furthermore, both mutants retain thymidine kinase (*tk*) expression allowing antiviral drug therapy (e.g., ganciclovir) to remain effective should systemic dissemination of virus occur.

Our experiments have shown that G207 efficiently infects, replicates within, and subsequently kills human and murine transitional cell carcinoma cell lines. All 5 cell lines proved to be remarkably susceptible to lysis following infection by G207 and NV1020, even at low MOIs. A correlation between infection efficiency and subsequent lysis was clearly demonstrated. We previously showed a correlation between more rapid cell division and cytolytic effect in human colorectal cancer cell lines (30). The current study, however, does not demonstrate a similar correlation. A correlation cannot be made between cell proliferation rate and susceptibility in the current study because all cell lines are rapidly dividing and, furthermore, all are sensitive to virus. NV1020 clearly demonstrated superior cytolytic effect at a lower MOI (0.1) in all three poorly differentiated human transitional cell carcinomas (J-82, SKUB, and T-24). The clinical implications of these findings are clear. In highly susceptible tumors, either viral preparation will likely prove efficacious. Patients with more resistant tumors or tumors of heterogeneous sensitivity will likely benefit from treatment with NV1020.

Bladder cancer is an ideal target for novel therapies because the ease of intravesical delivery allows the tumor to be exposed to large titers of vector. In addition, replication-competent oncolytic viruses, which selectively infect and replicate within rapidly dividing cells, are potentially very useful for bladder cancer because the umbrella cell layer (i.e., the luminal surface of the urothelium) of the bladder is not rapidly dividing and should therefore be resistant to infection and lysis. HSV is inactivated at low pH, and the relative acidity of the urine is of concern with this route of administration. These experiments demonstrate that these viruses maintain their infectivity and efficacy in this animal model when administered by intravesical instillation in PBS. It is likely that these agents will exert their therapeutic effect prior to inactivation by a low pH when administered with PBS as a buffer.

Data from the current experiments in a well-accepted, orthotopic, syngeneic model confirm safety, efficacy, and ease of delivery of oncolytic viral therapy for experimental treatment of bladder cancer. X-Gal staining demonstrated that G207 was capable of infecting tumor cells while sparing normal urothelial cells and normal tissues, including liver, kidney and brain. G207 was effective when administered by a single instillation in treating early (day 3) bladder tumors. Both viruses, when administered weekly for 3 weeks by intravesical instillation, were clearly more effective than control at both curing animals and reducing tumor burden. Intravesical BCG treatment has been evaluated by many investigators in this animal model (31, 32, 36, 37) and is currently the most effective treatment for patients with high-risk superficial bladder cancer (3, 7, 8). We have demonstrated that, in this animal model, both viruses are capable of comparable

efficacy to intravesical BCG. However, these oncolytic viruses will likely be more efficacious *in vivo* in humans because HSV are human viruses and human tumors are significantly more susceptible to their effects than murine tumors (Fig. 1) (28). Whether either virus will be more efficacious in human tumors *in vivo* than BCG, or whether either virus will have added effect when given with BCG in humans is unknown and will need to be resolved in human clinical studies.

Although both G207 and NV1020 are highly attenuated and replication restricted, therapeutic use of replicating viruses still raises concerns about viral proliferation and dissemination. In these experiments, treated animals continued to groom themselves and behave normally, no adverse events were noted, and there were no treatment-related deaths. Cured animals continued to gain weight while noncured animals lost weight toward the end of experiments because of tumor burden. Standard histology and histochemical analysis of multiple organs revealed no evidence of infection, inflammation, or other abnormality in animals treated with either virus. We analyzed normal bladder and kidneys because of possible vesico-ureteric reflux of virus, brain because of the natural neurotropism of herpes virus, ovary because of inherent rapid cell division in this organ, liver, myocardium, and lung. None of these tissues revealed positive results except for normal bladder from animals that were not inoculated with tumor, which showed scattered blue cells representing less than 1% of the umbrella cell layer of the bladder in 2 of 4 animals. We did not observe adverse events or treatment-related deaths following NV1020, and histology of multiple organs revealed no abnormalities. We and others have shown the safety of G207 and NV1020 in animal models with direct tumor injection, intravenous administration, and hepatic intra-arterial administration (21, 22, 28). The risk of extravasation of virus is low following intravesical instillation; but should it occur, our data from previous experiments indicates that it would be rapidly cleared from the bloodstream. Furthermore, studies of NV1020 in owl monkeys, the nonhuman primate most sensitive to wild-type HSV infection, have shown markedly attenuated pathogenicity and no transmission of virus to noninfected cage mates (16, 21).

A further margin of safety is provided by maintenance of the viral thymidine kinase (tk) gene. Unlike many other viruses, effective antiviral agents currently exist for the treatment of HSV infection because the tk gene allows the use of antiviral agents such as acyclovir for therapy if dissemination of virus and severe systemic toxicities are encountered. Furthermore, the existence of this gene allows for use of the prodrug ganciclovir for potential augmentation of anticancer activities. Phosphorylation of ganciclovir to the cytotoxic ganciclovir tri-phosphate allows tumor cells to be killed and is the basis for one gene therapy strategy under investigation that uses adenoviral vectors as the gene transfer vehicle for this gene to the tumor (38, 39). Other investigators have had some success in combining the use of ganciclovir with herpes simplex oncolytic therapy for treatment of brain tumors or colorectal cancers (40–42). These other studies, combined with the current studies demonstrating oncolytic HSV as effective single agents for the treatment of bladder cancer, encourage future investigations of combined therapy for bladder cancer.

We have demonstrated a selective, tumor-specific cytolytic effect in this orthotopic animal model of bladder cancer following intravesical administration of both G207 and NV1020. These viruses appear to be safe in the best available immunocompetent animal model of human bladder

cancer currently available. This report encourages further evaluation of intravesical oncolytic viral therapies for bladder cancer in clinical trials.

## REFERENCES

1. Landis, S.H., Murray, T., Bolden, S., and Wingo, P.A. (1999) Cancer statistics, 1999. *CA: Cancer J. Clin.* **49**, 8–31
2. Van der Meijden, A.P. (1998) Bladder cancer. *BMJ*, **317**, 1366–1369
3. Herr, H.W., Pinsky, C.M., Whitmore, W.F., Jr., Sogani, P.G., Oettgen, H.F., and Melamed, M.R. (1985) Experience with intravesical bacillus Calmette-Guerin therapy of superficial bladder tumors. *Urology* **25**, 119–123
4. Herr, H.W. (1997) Tumour progression and survival in patients with T1G3 bladder tumours: 15-year outcome. *Br. J. Urol.* **80**, 762–765
5. Lamm, D.L., Steg, A., Boccon-Gibod, L., Morales, A., Hanna, M.G., Jr., Pagano, F., Alfthan, O., Brosman, S., Fisher, H.A., and Jakse, G. (1989) Complications of Bacillus Calmette-Guerin immunotherapy: review of 2602 patients and comparison of chemotherapy complications. *Prog. Clin. Biol. Res.* **310**, 335–355
6. Lamm, D.L., Riggs, D.R., Traynelis, C.L., and Nseyo, U.O. (1995) Apparent failure of current intravesical chemotherapy prophylaxis to influence the long-term course of superficial transitional cell carcinoma of the bladder. *J. Urol.* **153**, 1444–1450
7. Lamm, D.L. (1995) BCG in perspective: advances in the treatment of superficial bladder cancer. *Eur. Urol.* **27**, 2–8
8. Pagano, F., Bassi, P., Milani, C., Piazza, N., Meneghini, A., and Garbeglio, A. (1992) BCG in superficial bladder cancer: a review of phase III European trials. *Eur. Urol.* **21**, 7–11
9. Hall, R. (1999) Neo-adjuvant cisplatin, methotrexate and vinblastine chemotherapy for muscle invasive bladder cancer: a randomised controlled trial. *Lancet*, **354**, 533–540
10. Ahlert, T., Sauerbrei, W., Bastert, G., Ruhland, S., Bartik, B., Simiontonaki, N., Schumacher, J., Hacker, B., Schumacher, M., and Schirmacher, V. (1997) Tumor-cell number and viability as quality and efficacy parameters of autologous virus-modified cancer vaccines in patients with breast or ovarian cancer [published erratum appears in *J. Clin. Oncol.* 1997 Jul; **15**(7):2763]. *JCO*, **15**, 1354–1366
11. Ockert, D., Schirmacher, V., Beck, N., Stoelben, E., Ahlert, T., Flechtenmacher, J., Hagmuller, E., Buchcik, R., Nagel, M., and Saeger, H.D. (1996) Newcastle disease virus-infected intact autologous tumor cell vaccine for adjuvant active specific immunotherapy of resected colorectal carcinoma. *Clin. Cancer Res.* **2**, 21–28

12. Van Pachterbeke, C., Tuynder, M., Brandenburger, A., Leclercq, G., Borras, M., and Rommelaere, J. (1997) Varying sensitivity of human mammary carcinoma cells to the toxic effect of parvovirus H-1. *Eur. J. Cancer* **33**, 1648–1653
13. Andreansky, S., Soroceanu, L., Flotte, E.R., Chou, J., Markert, J.M., Gillespie, G.Y., Roizman, B., and Whitley, R.J. (1997) Evaluation of genetically engineered herpes simplex viruses as oncolytic agents for human malignant brain tumors. *Cancer Res.* **57**, 1502–1509
14. Martuza, R.L., Malick, A., Markert, J.M., Ruffner, K.L., and Coen, D.M. (1991) Experimental therapy of human glioma by means of a genetically engineered virus mutant. *Science*. **252**(5007):854–856
15. Martuza R.L. Conditionally replicating herpes vectors for cancer therapy. (2000) *J. Clin. Invest.* **105**(7):841–846
16. Meignier, B. (1991). Genetically engineered attenuated herpes simplex viruses. *Rev. Infect. Dis.* **13**, S895–S897
17. Mineta, T., Rabkin, S.D., Yazaki, T., Hunter, W.D., and Martuza, R.L. (1995) Attenuated multi-mutated herpes simplex virus-1 for the treatment of malignant gliomas. *Nat. Med.* **1**, 938–943
18. Chou, J., Kern, E.R., Whitley, R.J., and Roizman, B. (1990) Mapping of herpes simplex virus-1 neurovirulence to gamma 134.5, a gene nonessential for growth in culture. *Science* **250**(4985):1262–1266
19. Goldstein, D.J., and Weller, S.K. (1988) Factor(s) present in herpes simplex virus type 1-infected cells can compensate for the loss of the large subunit of the viral ribonucleotide reductase: characterization of an ICP6 deletion mutant. *Virology* **166**, 41–51
20. Meignier, B., Longnecker, R., and Roizman, B. (1988) In vivo behavior of genetically engineered herpes simplex viruses R7017 and R7020: construction and evaluation in rodents. *J. Infect. Dis.* **158**, 602–614
21. Meignier, B., Martin, B., Whitley, R.J., and Roizman, B. (1990) In vivo behavior of genetically engineered herpes simplex viruses R7017 and R7020. II. Studies in immunocompetent and immunosuppressed owl monkeys (*Aotus trivirgatus*). *J. Infect. Dis.* **162**, 313–321
22. Advani, S.J., Chung, S.M., Yan, S.Y., Gillespie, G.Y., Markert, J.M., Whitley, R.J., Roizman, B., and Weichselbaum, R.R. (1999) Replication-competent, nonneuroinvasive genetically engineered herpes virus is highly effective in the treatment of therapy-resistant experimental human tumors. *Cancer Res.* **59**, 2055–2058
23. Soloway, M.S., deKernion, J.B., Rose, D., and Persky, L. (1973) Effect of chemotherapeutic agents on bladder cancer: a new animal model. *Surg. Forum*, **24**, 542–544

24. Soloway, M.S., Martino, C., Hyatt, C., and Marrone, J.C. (1978) Immunogenicity of N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide-induced bladder cancer. *Natl. Cancer Inst. Monogr.* 293–300
25. O'toole, C., Perlmann, P., Unsgaard, B., Moberger, G., and Edsmyr, F. (1972) Cellular immunity to human urinary bladder carcinoma. I. Correlation to clinical stage and radiotherapy. *Int. J. Cancer* **10**, 77–91
26. Pollack, M.S., Heagney, S.D., Livingston, P.O., and Fogh, J. (1981) HLA-A, B, C and DR alloantigen expression on forty-six cultured human tumor cell lines. *J. Nat. Cancer Inst.* **66**, 1003–1012
27. O'toole, C., Price, Z.H., Ohnuki, Y., and Unsgaard, B. (1978) Ultrastructure, karyology and immunology of a cell line originated from a human transitional-cell carcinoma. *Br. J. Cancer*, **38**, 64–76
28. Kooby, D.A., Carew, J.F., Halterman, M.W., Mack, J.E., Bertino, J.R., Blumgart, L.H., Federoff, H.J., and Fong, Y. (1999) Oncolytic viral therapy for human colorectal cancer and liver metastases using a multi-mutated herpes simplex virus type-1. *FASEB*, **13**, 1325–1334
29. Nusse, M., Beisker, W., Hoffmann, C., and Tarnok, A. (1990) Flow cytometric analysis of G1- and G2/M-phase subpopulations in mammalian cell nuclei using side scatter and DNA content measurements. *Cytometry*, **11**, 813–821
30. Giaretti, W., and Nusse, M. (1994) Light scatter of isolated cell nuclei as a parameter discriminating the cell-cycle subcompartments. *Methods Cell Biol.* **41**, 389–400
31. Shapiro, A., Ratliff, T.L., Oakley, D.M., and Aatalona, W.J. (1983) Reduction of bladder tumor growth in mice treated with intravesical Bacillus Calmette-Guerin and its correlation with Bacillus Calmette-Guerin viability and natural killer cell activity. *Cancer Res.* **43**, 1611–1615
32. Shapiro, A., Kelley, D.R., Oakley, D.M., Catalona, W.J., and Ratliff, T.L. (1984) Technical factors affecting the reproducibility of intravesical mouse bladder tumor implantation during therapy with Bacillus Calmette-Guerin. *Cancer Res.* **44**, 3051–3054
33. Toda, M., Rabkin, S.D., and Martuza, R.L. (1998) Treatment of human breast cancer in a brain metastatic model by G207, a replication-competent multimutated herpes simplex virus 1. *Hum. Gene Ther.* **9**, 2177–2185
34. Toda, M., Rabkin, S.D., Kojima, H., and Martuza, R.L. (1999) Herpes simplex virus as an in situ cancer vaccine for the induction of specific anti-tumor immunity. *Hum. Gene Ther.* **10**, 385–393

35. Yazaki, T., Manz, H.J., Rabkin, S.D., and Martuza, R.L. (1995) Treatment of human malignant meningiomas by G207, a replication-competent multmutated herpes simplex virus 1. *Cancer Res.* **55**, 4752–4756
36. Mcaveney, K.M., Gomella, L.G., and Lattime, E.C. (1994) Induction of TH1- and TH2-associated cytokine mRNA in mouse bladder following intravesical growth of the murine bladder tumor MB49 and BCG immunotherapy. *Cancer Immunol Immunother.* **39**, 401–406
37. Shin, J.S., Park, J.H., Kim, J.D., Lee, J.M., and Kim, S.J. (1995) Induction of tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) mRNA in bladders and spleens of mice after intravesical administration of bacillus Calmette-Guerin. *Clin. Exp. Immunol.* **100**, 26–31
38. Li, Q., Kay, M.A., Finegold, M., Stratford-Perricaudet, L.D., and Woo, S.L.C. (1993) Assessment of recombinant adenoviral vectors for hepatic gene therapy. *Hum. Gene Ther.* **4**, 403–409
39. Morris, J.C., and Wildner, O. (2000) Therapy of head and neck squamous cell carcinoma with an oncolytic adenovirus expressing HSV-tk. *Molecular Therapy: the Journal of the American Society of Gene Therapy* **1**, 56–62
40. Carroll, N.M., Chase, M., Chiocca, E.A., and Tanabe, K.K. (1997) The effect of ganciclovir on herpes simplex virus-mediated oncolysis. *Journal of Surgical Research* **69**, 413–417
41. Kramm, C.M., Rainov, N.G., Sena-Esteves, M., Barnett, F.H., Chase, M., Herrlinger, U., Pechan, P.A., Chiocca, E.A., and Breakefield, X.O. (1996) Long-term survival in a rodent model of disseminated brain tumors by combined intrathecal delivery of herpes vectors and ganciclovir treatment. *Hum. Gene Ther.* **7**, 1989–1994
42. Todo, T., Rabkin, S.D., and Martuza, R.L. (2000) Evaluation of ganciclovir-mediated enhancement of the antitumoral effect in oncolytic, multmutated herpes simplex virus type 1 (G207) therapy of brain tumors. *Cancer Gene Ther.* **7**, 939–946

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**Table 1****Characteristics of cell lines examined with double times and s-phase fraction**

Cell line	Double time (hours)	s-phase fraction	Transitional cell carcinoma cell type
RT-4	30.5	22.3	Human well differentiated
T-24	28	27.7	Human poorly differentiated
SKUB	25	29.6	Human poorly differentiated
J-82	24	34.2	Human poorly differentiated
MBT-2	22	51	Syngenic mouse



**Table 2**

**Results of intravesical treatment (administered weekly for three weeks) of orthotopic bladder cancer (MBT-2) in a syngenic (C3H/HeJ) animal model.**

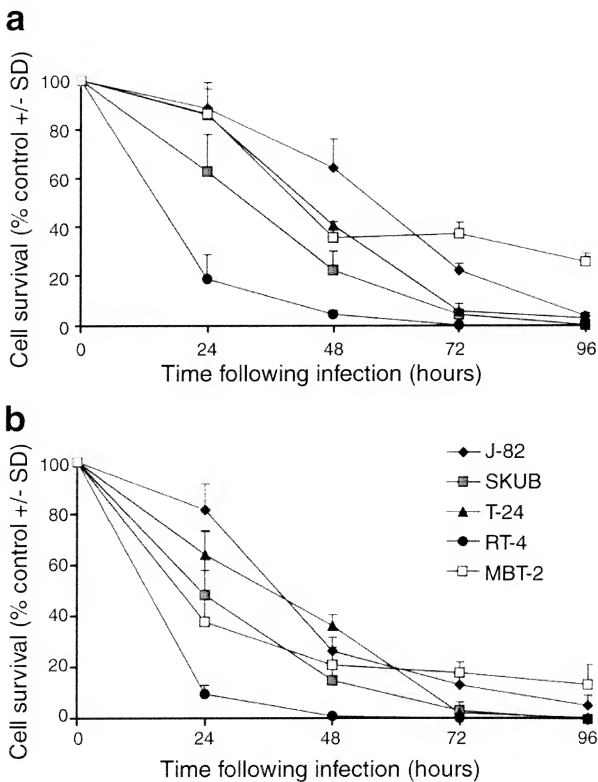
	<b>Number of animals</b>	<b>Weight loss Mean +/- (SD) g<sup>1</sup></b>	<b>Animals with Tumor<sup>2</sup></b>	<b>Tumor burden Mean +/- (SD) mg<sup>3</sup></b>
<b>Control</b>	11	2.00 (0.67)	10	110 (7)
<b>BCG</b>	12	1.00 (0.28)	6	72 (120)
<b>G207</b>	13	1.56 (0.77)	5	35 (50)
<b>NV1020</b>	12	1.33 (0.14)	2	13 (30)

<sup>1</sup> Weight loss calculated in weight from day 0 to day 21 when the experiment was terminated

<sup>2</sup> Animals with tumor include animals with tumors demonstrable on macroscopic or microscopic (histologic) examination at necropsy on day 21.

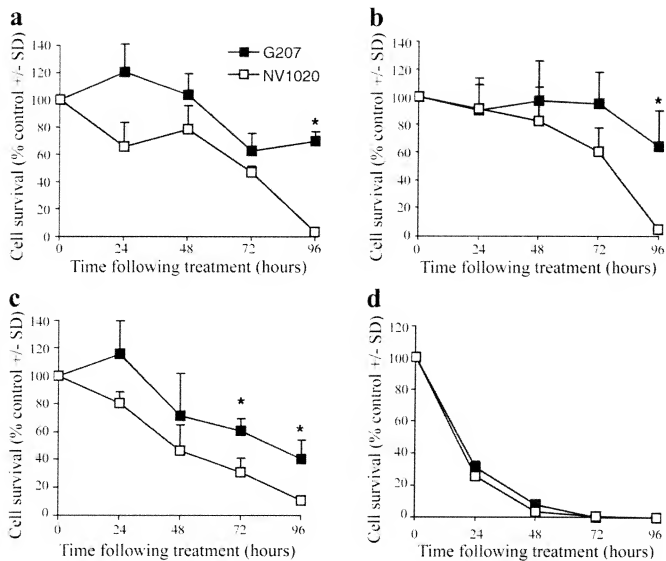
<sup>3</sup> Tumor burden includes weight of tumor and empty bladder at necropsy on day 21.

Fig. 1



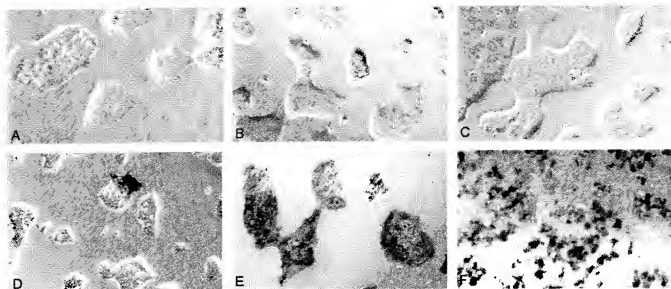
**Figure 1.** Effect on cell viability of transitional cell carcinoma cell lines *in vitro* after infection at MOI 1 of G207 (a) and NV1020 (b).

**Fig. 2**



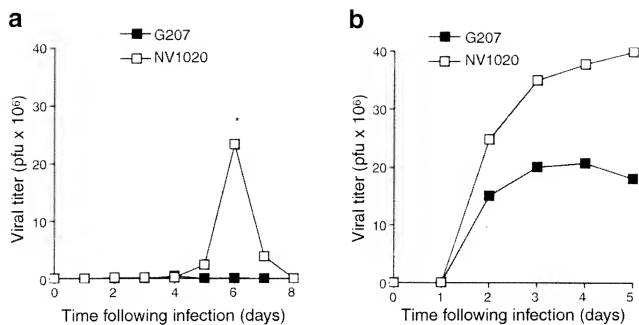
**Figure 2.** Effect of G207 (■) and NV1020 (□) in direct comparison in the human cell lines. MOI 0.1 (a) T-24; (b) J-82; (c) SKUB; (d) RT-4. \*  $P < 0.05$ .

**Fig. 3**



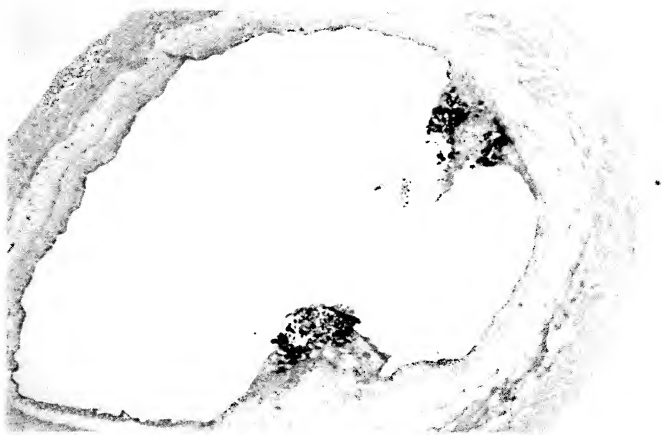
**Figure 3.** X-Gal staining of RT-4 cells following G207 treatment (MOI 0.01): Control at 24 (A), 48 (B), and 72 h (C). Treated wells at 24 (D), 48 (E), and 72 h (F).

**Fig. 4**



**Figure 4.** Viral titer for J-82 (a) and RT-4 (b) at MOI 0.01 for G207 (■) and NV1020 (□). \*  $P < 0.05$ .

**Fig. 5**



**Figure 5.** X-Gal staining of day 8 bladder tumors 48 h after treatment with  $1 \times 10^7$  PFUs of G207.